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Stimulation of ionotropic glutamate receptors activates transcription factor NF-κB in primary neurons

(kainate/depolarization/major histocompatibility complex class I)

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ABSTRACT **L-Glutamate is the most common excitatory neurotransmitter in the brain and plays a crucial role in neuronal plasticity as well as in neurotoxicity. While a large body of literature describes the induction of immediate-early genes, including c-*fos*, *fosB*, c-*jun*, *junB*, *zif/268*, and *krox* genes by glutamate and agonists in neurons, very little is known about preexisting transcription factors controlling the induction of such genes. This prompted us to investigate whether stimulation of glutamate receptors can activate NF-κB, which is present in neurons in either inducible or constitutive form. Here we report that brief treatments with kainate or high potassium strongly activated NF-κB in granule cells from rat cerebellum. This was detected at the single cell level by immunostaining with a monoclonal antibody that selectively reacts with the transcriptionally active, nuclear form of NF-κB p65. The activation of NF-κB could be blocked with the antioxidant pyrrolidine dithiocarbamate, suggesting the involvement of reactive oxygen intermediates. The data may explain the kainate-induced cell surface expression of major histocompatibility complex class I molecules, which are encoded by genes known to be controlled by NF-κB. Moreover, NF-κB activity was found to change dramatically in neurons during development of the cerebellum between days 5 and 7 after birth.**

The activity of the inducible, ubiquitous transcription factor NF-κB is predominantly regulated at the posttranslational level (1–4). In most cell types, NF-κB mediates an immediate-early pathogen response by coordinately initiating the transcription of numerous genes encoding cytokines, chemokines, hematopoietic growth factors, cell adhesion molecules, major histocompatibility complex (MHC) class I molecules, and acute phase proteins. Stimuli that activate the preexisting factor mostly represent pathogenic conditions, such as viruses, bacteria, UV light, and the inflammatory cytokines interleukin 1 and tumor necrosis factor. The inducible form of NF-κB resides in the cytoplasm and contains an additional inhibitory subunit called IκB. Activation of NF-κB in response to various stimuli involves phosphorylation of the inhibitory subunit IκB-α on serines 32 and 36 (5, 6). This does not release IκB-α from the dimerized DNA-binding subunits of NF-κB but dramatically enhances the turnover of the inhibitor. After degradation of IκB-α by the proteasome (7, 8), the released NF-κB is transported into the nucleus, binds to DNA, and initiates transcription by recruiting the basal transcription factors TFIIB and TBP and the positive coactivator 1 (9).

cDNA cloning has identified five members of the NF-κB family of DNA-binding subunits in higher vertebrates (1–4), which can hetero- and homodimerize to a certain extent. This yields dimers of distinguishable DNA-binding specificity, transcriptional potential, and IκB susceptibility. Likewise, there are a variety of IκB proteins (10, 11). One frequently observed dimer combination contains the DNA-binding subunits p50 and p65 (RelA). It is also found in many different regions of the nervous system in an inducible form (12, 13). Inducible NF-κB was found in primary astrocytes, glioblastoma cells (14, 15), and microglial cells where NF-κB can be activated during experimental autoimmune encephalomyelitis (16). Using a monoclonal antibody (α-p65Mab), which selectively binds the activated nuclear form of NF-κB, we were surprised to find that both cultured primary cortical and hippocampal as well as neurons in cryosections from the brains of undiseased rodents contain activated NF-κB in their nuclei (17, 18). This was also evident from increased human immunodeficiency virus type 1 long terminal repeat-controlled reporter gene activity (18, 19). The activated NF-κB was restricted to a subset of neurons, suggesting that in neurons a physiological, endogenous stimulus was controlling the activity of the transcription factor.

L-Glutamate is the most common neurotransmitter in the brain and causes a depolarization of neurons by binding to multisubunit ionotropic receptors (20–23). Glutamate-gated ion channels are pharmacologically classified by the action of the agonists *N*-methyl-D-aspartate (NMDA), DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate. Both AMPA and kainate exhibit significant affinity for genetically distinct receptor types. However, there is no agonist or antagonist available that can differentiate between the receptors, which is why they are referred to as AMPA/kainate or non-NMDA receptors. Among various other reactions, ligand binding to these receptors was reported to cause production of reactive oxygen intermediates (ROIs) in neurons (24).

NF-κB appears to be a good candidate for a mediator of glutamate-induced immediate-early gene transcription. First, the transcription factor is present in both activated and inducible form in neurons of the hippocampus, which are known to be responsive to glutamate (19). Second, NF-κB is activated posttranslationally, a process that frequently requires production of ROIs (25–27). Here we report that glutamate and agonists activate transcription factor NF-κB in primary neurons.

MATERIALS AND METHODS

Immunostaining of Brain Tissue. Wistar rats were decapitated at various times after birth. Brains were removed and cerebella were dissected. Tissue was embedded in OTC compound (Miles-Bayer, Leverkuren, F.R.G.) and quick frozen in liquid nitrogen. Cryostat sections (8 μm) were cut with a Jung cryostat (Leica, Heidelberg, F.R.G.) and mounted on gelatin-

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Abbreviations: MHC, major histocompatibility complex; PDTC, pyrrolidine dithiocarbamate; ROI, reactive oxygen intermediate; NMDA, *N*-methyl-D-aspartate; AMPA, DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAPI, 4′,6-diamidino-2-phenylindole.

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coated slides. Immunohistochemistry was performed essentially as described (13, 17, 18). Briefly, slides were fixed for 5 min in methanol at −20°C, washed, and incubated with 5% goat serum. α-p65Mab was used at a dilution of 1:50 (20 μg/ml). The bound monoclonal antibody was detected with a biotinylated anti-mouse IgG followed by decoration with Cy3-conjugated streptavidin (Dianova, Hamburg, F.R.G.). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim). Micrographs were taken with an Axiovert photomicroscope (Zeiss) equipped with epifluorescence. Ten rats from different litters were analyzed.

Primary Culture and Drug Treatments. Granule cells were prepared as described by Gallo *et al.* (31) with slight modification. Briefly, cerebella from postnatal day 7 pups were removed, freed of meninges, and chopped with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, Surrey, U.K.) in two orthogonal sections, which were treated with trypsin (0.25 mg/ml) and DNase I (12.8 μg/ml). Cells were plated on poly(L-lysine)-coated chamber slides (Nunc) in basal modified Eagle's medium (Seromed, Berlin) containing 10% heat-treated fetal calf serum, gentamicin (100 μg/ml), and 25 mM KCl. Cytosine arabinonucleoside (10 μM) was added 18 h after plating to suppress growth of nonneuronal cells. Cultures contained <5% nonneuronal cells, as detected by immunofluorescence analysis using neuronal marker antibodies: anti-neurofilament 200 (Sigma), anti-neuron-specific enolase (Dianova, Hamburg, F.R.G.), and the astrocytic marker anti-glial fibrillary acidic protein (Boehringer Mannheim). Neurons were exposed to excitatory amino acids or KCl in Tris-buffered control salt solution (CSS) (32) containing 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM Tris·HCl (pH 7.4), 15 mM D-glucose. Before a 5-min treatment with 100 μM kainate, cells were washed three times with CSS. Cells were preincubated for 10 min with either 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 10 μM pyrrolidine dithiocarbamate (PDTC) before treatment with kainate. After treatments, cells were washed with CSS and incubated with complete medium for 45 min at 37°C. Control cells received identical incubations and washings with CSS. Cells were fixed for 2 min in ethanol and for 5 min in 3.7% formaldehyde. Indirect immunofluorescence was performed as described above. The sequence of the peptide used for antibody competition is described elsewhere (17). Kainate and CNQX were obtained from Research Biochemicals (Natick, MA), and the ammonium salt of PDTC was from Sigma. The depolarizing treatment was performed for 5 min with 100 mM KCl, which replaced an equimolar amount of NaCl in the CSS buffer. The rat MHC class I RT1A^a was detected by a monoclonal antibody (Serotec, Oxford). Detection of bound antibody was performed with indirect immunofluorescence as described above.

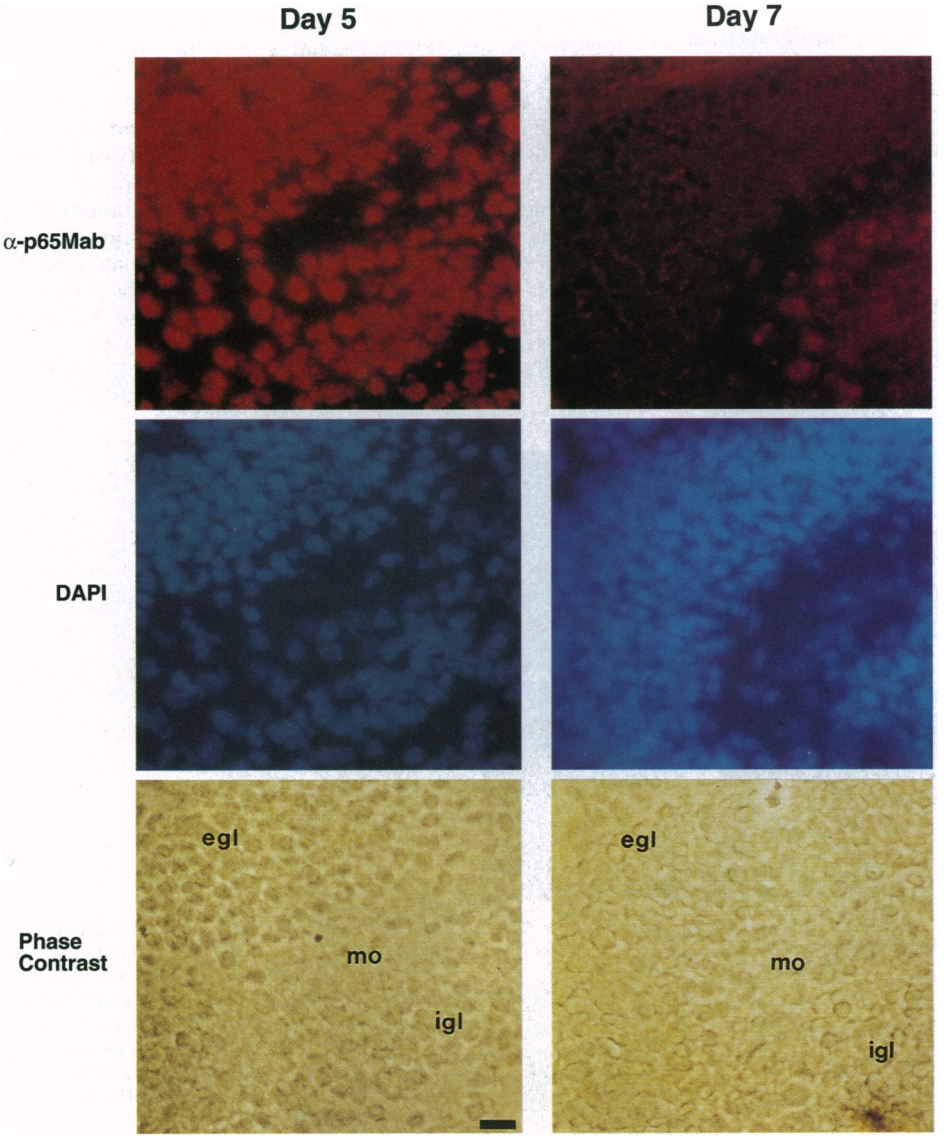

FIG. 1. The state of NF-κB activity in developing cerebellum. Cryosections from cerebellum at day 5 (*Left*) and day 7 (*Right*) were analyzed by activated NF-κB indirect immunofluorescence labeling using the monoclonal antibody α-p65Mab (*Top*). (*Middle*) Same sections stained for nuclear DNA by DAPI. (Bottom) Phase-contrast micrographs of the same sections. egl, External granular layer; mo, molecular layer; igl, internal granular layer. Purkinje cells can be identified in the molecular layer by their larger nuclei. (Bar = 50 μm.)

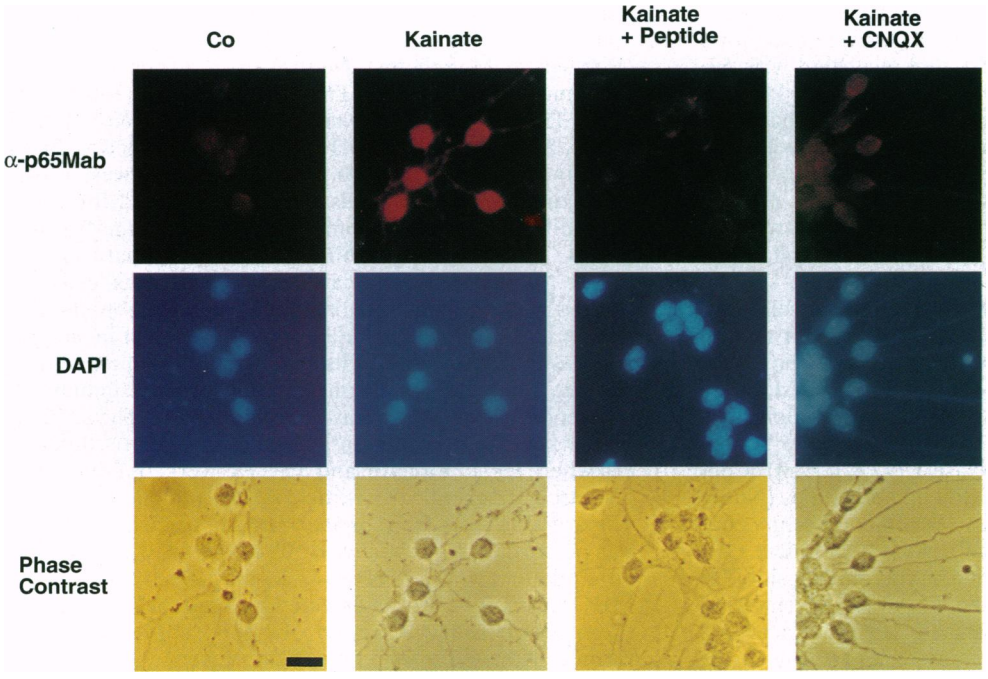

FIG. 2. Effect of kainate on activity of NF-κB in primary cerebellar granule cells. (*Top*) Indirect immunofluorescence analysis of granule cell cultures for p65 NF-κB immunoreactivity using the activity-specific monoclonal α-p65Mab. (*Middle*) DNA staining of cultures using DAPI. (*Bottom*) Phase-contrast micrographs. Granule cells were prepared from cerebella of 8-day-old pups and analyzed after 7 days in culture. First column, mock-treated control cells; second column, 5-min treatment with 100 μM kainate; third column, immunostaining of kainate-stimulated cells with peptide-preabsorbed α-p65Mab; fourth column, 10-min pretreatment with 50 μM CNQX followed by stimulation with 100 μM kainate. (Bar = 50 μM.)

RESULTS

Neuronal NF-κB Activity in the Developing Rat Cerebellum Decreases Between Days 5 and 7. We investigated the potential of glutamate to stimulate NF-κB. An initial complication was that freshly cultured neurons from 1- to 5-day-old rats contained already activated NF-κB in their nuclei, as determined by immunofluorescence staining of primary granule cell cultures with the activity-specific monoclonal antibody α-p65Mab (data not shown). Consistent with this finding, cryosections of cerebellum from 5-day-old rats revealed a strong nuclear p65 immunostaining in neurons from all three cell layers of the cerebellum (Fig. 1 *Top Left*). The immunostaining was strongly quenched when α-p65Mab was preincubated with the peptide epitope (data not shown; see Fig. 2). In contrast to day 5, α-p65Mab immunostaining was strongly reduced at day 7 in the external layer and no longer concentrated in nuclei (Fig. 1 *Right*). The p65 immunostaining was also reduced in the internal granular layer, where only very few cells remained positive. Within the molecular layer, selected Purkinje neurons were the only cells left with a strong nuclear immunostaining (unpublished data). This shows that the activity of NF-κB is highly regulated in the developing cerebellum of rats.

Activation of NF-κB in Granule Cells by Glutamate Receptor Stimulation. After testing many different conditions, low levels of α-p65Mab immunostaining were obtained upon prolonged (5–7 days *in vitro*) culture of granule cells, which were derived from 6- to 8-day-old rats (Figs. 2 and 3 *Top*, first columns). The primary neurons showed only a faint cytoplasmic p65 immunostaining around their nuclei. These culture

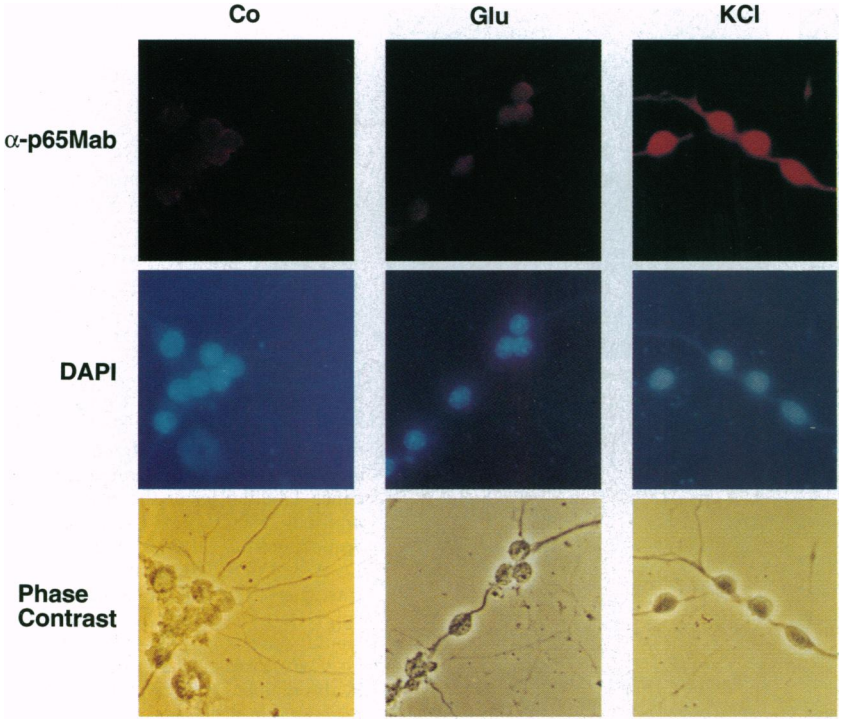

FIG. 3. Effect of glutamate and high KCl on activity of NF-κB in cerebellar granule cells. First column, mock-treated control granule cells; second column, cells treated for 5 min with 500 μM L-glutamate; third column, cells treated for 5 min with 100 mM KCl. Cultures were analyzed 45 min later for activated p65 NF-κB by indirect immunofluorescence analysis. See legends to Figs. 1 and 2 for further details.

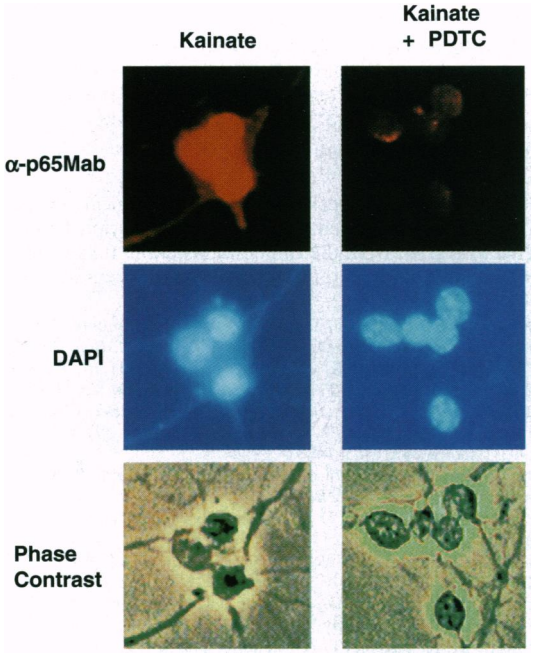

FIG. 4. Effect of the antioxidant PDTC on kainate-mediated activation of NF-κB. (*Left*) Granule cells were treated for 5 min with 100 μM kainate. (*Right*) Pretreatment for 10 min with 10 μM PDTC followed by stimulation with 100 μM kainate. Cultures were analyzed for activated p65 by indirect immunofluorescence staining as described in legends to Fig. 1 and 2.

conditions were used throughout subsequent analyses. One class of widespread glutamate receptors is activated by the agonist kainate (20). After a 5-min pulse with 100 μM kainate, α-p65Mab immunoreactivity was strongly enhanced in nuclei of granule cells 45 min later (Fig. 2, compare first and second columns). Immunostaining in the cytoplasm of soma and neurites was also strongly increased, indicating the release of NF-κB from its cytoplasmic form prior to nuclear translocation. The immunostaining was specific because it was strongly quenched when the α-p65Mab was preincubated with the p65 peptide epitope (third column). The effect of kainate was suppressed by preincubation of cells with the antagonist CNQX at 50 μM (fourth column). These data provide pharmacological evidence for an involvement of kainate/AMPA-dependent glutamate receptors in NF-κB activation. A 5-min pulse with 500 μM L-glutamate could also increase nuclear α-p65Mab immunoreactivity but to a much lower level than observed with kainate (Fig. 3 *Top*, second column). A condition known to indirectly activate NMDA-type glutamate receptors in granule cells is depolarizing treatment with 100 mM KCl (28, 30, 31). As shown in Fig. 3 (third column), a 5-min pulse with 100 mM KCl strongly induced α-p65Mab reactivity 45 min later to a level similar to that seen with kainate. Treatment with 300 μM NMDA in the presence of 10 μM glycine had no effect (data not shown). This might be due to a Mg^{2+} blockade of the NMDA receptor (21, 30, 31). Depolarization with KCl is known to relieve the Mg^{2+} block of the NMDA receptor, which can now respond to released glutamate. In conclusion, these data show that stimulation of glutamate neurotransmitter receptors can activate transcription factor NF-κB in primary neurons. This involves receptors of the kainate/AMPA type and, presumably, also of the NMDA type. None of the brief (5 min) treatments with glutamate or agonists caused any visible neurotoxic effects within 6 h. Neurotoxic effects after a longer period cannot be excluded. A 24-h treatment of cerebellar granule cells with glutamate or kainate caused extensive neuronal death, as reported earlier (32, 33).

Activation of NF-κB by Kainate Is Blocked by the Antioxidant PDTC. Stimulation of ionotropic glutamate receptors is a condition known to generate ROIs (24). In many different cell types, ROIs can activate NF-κB and there is pharmacological and molecular genetic evidence to strongly suggest a requirement of ROIs for NF-κB activation by many other inducers (25–27). Therefore, we tested whether NF-κB activation by glutamate receptor stimulation is suppressed by the antioxidant PDTC, a potent inhibitor of ROI production and NF-κB activation in other cell types (34). While granule cells strongly activated NF-κB after stimulation with 100 μM kainate for 5 min (Fig. 4 *Left*), granule cells preincubated for 10 min with 10 μM PDTC could no longer efficiently activate the transcription factor (Fig. 4 *Right*). PDTC inhibition was also seen with KCl-stimulated granule cells (data not shown). These data provide pharmacological evidence for involvement of ROIs in the activation of NF-κB by ionotropic glutamate receptors.

Activation of NF-κB by Kainate Coincides with the Expression of MHC Class I Genes in Granule Cells. We were interested in determining whether the appearance of α-p65-Mab immunoreactivity correlates with induction of a well-characterized NF-κB-regulated gene in cultured granule cells. In many cell types, including neuroblastoma lines (35), MHC class I and β_2-microglobulin genes are known to be coordinately upregulated by NF-κB in response to cytokines (1–4, 36). Since it is known that primary neurons do not express detectable levels of MHC class I under normal culture conditions (37), we wanted to determine whether kainate is capable of inducing MHC class I expression. Using a monoclonal antibody with specificity for rat $RT1A^a$, only a faint immunoreactivity for the class I molecule was detected in cultured granule cells under control conditions (Fig. 5 *Left*). Exposure of cells to 100 μM kainate for 5 min, a condition activating NF-κB (see Fig. 2), caused a strongly enhanced immunostaining for $RT1A^a$, as detected 6 h later (Fig. 5 *Right*).

DISCUSSION

NF-κB is posttranslationally activated by proteolytic removal of the inhibitory subunit IκB from a latent cytoplasmic form (1–4). This leads to the appearance of NF-κB-specific DNA-binding activity, which can be detected by electrophoretic mobility shift assays in cell extracts. However, this method does not allow monitoring NF-κB activation in individual cells. To study NF-κB activation at the cellular level in a small number of primary cells and in tissue sections, we have developed a monoclonal antibody (α-p65Mab) (17). It binds a nuclear location signal sequence on the transactivating p65 subunit, which is shielded by IκB-α in the latent cytoplasmic form but exposed on the transcriptionally active, nuclear form of NF-κB (38). The gene encoding p65 is constitutively expressed and its expression and promoter activity have not been found to be enhanced upon stimulation of cells (39). Likewise, the protein levels of p65 do not change detectably during activation of NF-κB by various stimuli, as detected by Western blotting (40). Hence, it is very unlikely that a dramatically increased nuclear p65 immunostaining, as observed here in tissue sections and primary cell cultures, is due to synthesis of active p65 rather than loss of IκB from preexisting p65.

Use of the activity-specific α-p65Mab and polyclonal antibodies has shown that subsets of neurons in the cerebral cortex and hippocampus of rats and mice contain activated NF-κB in their nuclei (17, 18). Mobility-shift assays mainly detected inducible NF-κB in total homogenates from the same brain tissues, most likely because glial cells, which contain inducible NF-κB, constitute the major cell population in brain tissues (12, 13). In the present study, we have observed that the cerebellum from newborn rats up to day 5 also contains high levels of activated NF-κB in nuclei of most neurons from the

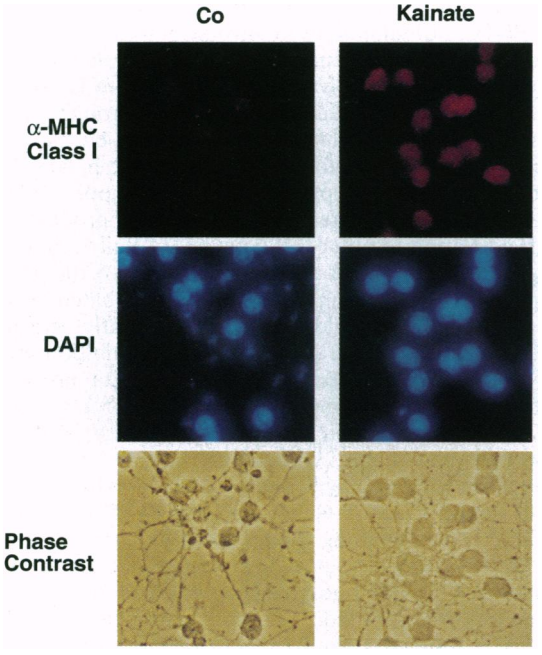

FIG. 5. Effect of kainate on expression of MHC class I molecules in cerebellar granule cells. Primary neurons were analyzed for expression of MHC class I molecules by indirect immunofluorescence analysis. (*Left*) Unstimulated granule cells. (*Right*) Treatment for 5 min with 100 μM kainate. See legend to Fig. 2 for further details. MHC class I expression was followed for 6 h after the kainate pulse.

internal and external granular as well as molecular layers. Only 1–2 days later, a sharp drop of NF-κB activity occurred. This was not due to the downregulation of p65 protein levels since glutamate agonists strongly induced NF-κB in cultured cells from this postnatal stage. Glutamate is discussed as a developmental stimulus for the cerebellum (41, 42). It is therefore tempting to speculate that some changes in gene expression during cerebellar development could be governed by glutamate-induced gene expression involving NF-κB as one transcription factor. This would indicate that NF-κB in neurons is not only involved in the response to pathogenic stimuli but would also participate in the normal physiology and development of the nervous system.

So far, it was unknown which preexisting transcription factors mediate the signaling from the membrane-bound ionotropic glutamate receptors to the nucleus of neurons. Here, we have identified NF-κB as a factor that translocates to the nuclei of primary neurons upon stimulation with glutamate, kainate, and depolarization. While NF-κB is activated in most cell types with signals that represent pathological conditions—for instance, in microglia during experimental autoimmune encephalomyelitis (16)—neurons seem special in that the factor is induced by nonpathological endogenous signals, such as neurotransmitters. The signaling pathway by which glutamate receptors trigger NF-κB activation appears to be related to that used by other NF-κB inducers in other cell types. Activation of various glutamate receptors induces oxidative stress (24). Increased ROI production is also seen with many other NF-κB stimuli (25–27), such as tumor necrosis factor, interleukin 1, phorbol esters, and amyloid β peptide (43). The inhibitory effect of many antioxidants and catalase overexpression, as well as the NF-κB-activating effect of H_2O_2 in several cell lines (25–27), suggested an involvement of H_2O_2 as common messenger in the activation of NF-κB (26). Here we provide pharmacological evidence by inhibitory treatment with an antioxidant that the ROIs produced in response to glutamate receptor agonists are necessary for NF-κB activation in neurons. Glutamate plays an important role in both neuronal plasticity and neurodegeneration (20). Hence, it will be interesting to discern the contribution of NF-κB-controlled gene expression to each of these phenomena. Kainate-inducible genes, which were recently isolated from the hippocampus by differential cDNA screening (44), are likely to include many NF-κB-regulated genes.

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